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14. ABSTRACT

Organophosphorus esters (OP) are highly toxic poisons used as chemical nerve agents and as pesticides. It is generally agreed that the toxicity from high dose OP exposure involves inhibition of acetyl cholinesterase. The role of other proteins in the toxicity of OP is unknown. Our hypothesis is that several proteins become modified after exposure to OP and that the biological actions of OP are not explained by inhibition of acetylcholinesterase alone. We are using mass spectrometry to identify proteins modified by exposure to OP. A new motif for OP binding is beginning to emerge from our work. We are seeing a pattern of covalent OP binding to tyrosine where the tyrosine is adjacent to an arginine or lysine. We have identified tyrosine 411 on the surface of human albumin and tyrosine 238 of human transferrin as OP-binding sites. Our results are relevant to diagnosis of OP exposure. The mass spectrometry methods we have developed are rapid and simple, but expensive. The new information from our mass spectrometry results can be used to develop antibody based dipstick assays to diagnose OP exposure.

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Bin Li, Lawrence M. Schopfer, Steven H. Hinrichs, Patrick Masson, Oksana Lockridge (2007) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay for organophosphorus toxicants bound to human albumin at Tyr 411. *Analytical Biochemistry*. In press.

ABBREVIATIONS

amu	atomic mass unit
CHCA	alpha-cyano 4-hydoxycinnamic acid
DFP	diisopropylfluorophosphate
FP-biotin	biotin-tagged organophosphorus agent; 10-fluoroethoxyphosphinyl-N-biotinamidopentyldecanamide
HPLC	high performance liquid chromatography
LC/MS/MS	liquid chromatography coupled to tandem mass spectrometry
m/z	mass to charge ratio
OP	organophosphorus toxicant
PVDF	polyvinylidene fluoride membrane for protein blots
SDS	sodium dodecyl sulfate

INTRODUCTION

Organophosphorus esters (OP) are highly toxic poisons used as chemical nerve agents and as pesticides. It is generally agreed that the toxicity from high dose OP exposure involves inhibition of acetylcholinesterase. The role of other proteins in the toxicity of OP is unknown. Our hypothesis is that several proteins become modified after exposure to OP and that the biological actions of OP are not explained by inhibition of acetylcholinesterase alone. The basis for this hypothesis is our finding that a mouse treated with a biotin-tagged nerve agent analog, called FP-biotin, acquires many biotin-tagged proteins in blood. Support for our hypothesis comes from the work of toxicologists who find that poisoning symptoms are different for each OP when the dose of OP is nonlethal (Moser, 1995). For example, fenthion decreases motor activity but does not alter the tail-pinch response, whereas parathion does not lower motor activity but does decrease the tail-pinch response. If all OP reacted only with acetylcholinesterase and with no other proteins, then all OP should give the same toxic signs.

We are using mass spectrometry to identify proteins modified by exposure to OP. Until now it has been thought that the OP-reactive proteins are exclusively serine hydrolases, characterized by serine in the active site. Our work shows that this is too narrow a view, and that OP also modify proteins without an active site serine. A new motif for OP binding is beginning to emerge from our work. We are seeing a pattern of covalent OP binding to tyrosine where the tyrosine is adjacent to an arginine or lysine.

Our results are relevant to diagnosis of OP exposure. The mass spectrometry methods we have developed are rapid and simple, but expensive. The new information from our mass spectrometry results can be used to develop antibody based dipstick assays to diagnose OP exposure.

APPROVED STATEMENT OF WORK

Task 1. Identify proteins labeled by FP-biotin

1.1. Identify FP-biotin labeled proteins in human blood
reported 20 April 2006

1.2. Identify the amino acid covalently attached to FP-biotin in each FP-biotinylated human protein.
reported 20 October 2006

1.3. Identify FP-biotin labeled proteins in mouse blood
reported 21 July 2006

1.4. Identify the amino acid covalently attached to FP-biotin in each FP-biotinylated mouse protein.
reported annual report 24 December 2006

1.5. Inject mice with FP-biotin and identify the FP-biotinylated proteins in mouse blood.

Task 2. Identify proteins labeled by DFP

2.1. Label pure human butyrylcholinesterase with DFP, isolate the DFP-labeled tryptic peptide, and identify the characteristic ion fragments produced by collision induced dissociation.

2.2. Label pure human albumin with DFP, isolate the DFP-labeled tryptic peptide, and identify the characteristic ion fragments produced by collision induced dissociation.
reported July 21, 2006; paper in press

2.3. Label pure bovine trypsin with DFP, isolate the DFP-labeled tryptic peptide, and identify the characteristic ion fragments produced by collision induced dissociation.

2.4. Identify the proteins in human blood that bind DFP.

2.5. Identify the proteins in mouse blood that bind DFP.

2.6. Inject mice with DFP and identify the DFP-labeled proteins in mouse blood.

TASK 1

Task 1. Identify proteins labeled by FP-biotin
1.1. Identify FP-biotin labeled proteins in human blood
1.2. Identify the amino acid covalently attached to FP-biotin in each FP-biotinylated human protein.

Relation to statement of work. Results for tasks 1.1 and 1.2 are reported.

Tasks 1.1 and 1.2

OP-binding proteins in human plasma: a new motif for OP binding to tyrosine

Summary
OP binding proteins in human plasma were identified after labeling human plasma with FP-biotin. The FP-biotinylated proteins were purified on immobilized avidin beads, and separated by gel electrophoresis. Coomassie stained bands were digested with trypsin and analyzed by LC/MS/MS. Proteins were identified by analysis of fragmentation patterns using Mascot software. Thirty-one proteins were identified. Eight proteins have the consensus sequence for serine hydrolases. Two proteins, albumin and transferrin, bind OP to tyrosine. Albumin binds OP to Tyr 411, while transferrin binds OP to Tyr 238. Twenty-one proteins have an unknown OP binding site. Samples for mass spectrometry analysis were prepared by a second method, by purifying FP-biotinylated peptides on monomeric avidin beads. This method identified FP-biotinylated peptides where the OP was covalently bound to tyrosine or serine, but did not identify the parent protein. A new motif for OP binding to tyrosine is beginning to emerge.

Introduction

Diagnosis of OP exposure relies on symptoms of cholinergic toxicity and on laboratory tests of cholinesterase activity in blood. A few specialized laboratories have gas chromatography/mass spectrometry equipment to look for OP metabolites in blood and urine. The fluoride reactivation method introduced by the TNO Prins Maurits Laboratory in the Netherlands (Polhuijs et al., 1997; Degenhardt et al., 2004) recovers OP covalently attached to protein and then analyzes the released OP by gas chromatography/mass spectrometry. Another method to analyze OP exposure is to measure the mass of the

butyrylcholinesterase active site peptide (Van Der Schans et al., 2004; Tsuge and Seto, 2006). The assumption in these assays is that butyrylcholinesterase in plasma and acetylcholinesterase in red blood cells are the only proteins modified by OP. It was a surprise therefore, when we treated mice with a biotin-tagged OP called FP-biotin, and found that the majority of the labeled protein was albumin (Peeples et al., 2005). Many additional proteins also became labeled. We set out to identify these additional proteins using mass spectrometry.

Materials and Methods

Materials. FP-biotin was custom synthesized in the laboratory of Dr. Charles Thompson at the University of Montana, Missoula, MT. FP-biotin was dissolved in methanol and stored at -80˚C. Immobilized avidin beads (Pierce #20219) and immobilized monomeric avidin beads (Pierce #20228) were used to purify FP-biotinylated proteins as well as FP-biotinylated peptides. Affi-gel blue gel (BioRad #153-7301) is a crosslinked agarose bead with covalently attached Cibacron Blue F3GA dye used for depletion of albumin from plasma. Proteome Partitioning Kit, ProteomeLab IgY-12 High Capacity in Spin Column format contains IgY antibodies directed against the 12 most abundant proteins in human plasma (Beckman Coulter #A24331 S0510903). Microwave Blue (Protiga, Frederick, MD #P1002-MWB) was used to stain polyacrylamide gels.

Porcine pepsin (Sigma, St. Louis, MO; P6887 from porcine gastric mucosa) was dissolved in 10 mM HCl to give a 1 mg/ml solution and stored at -80˚C. Porcine trypsin (Promega, Madison, WI; V5113 sequencing grade modified trypsin) had a concentration of 0.4 µg/µl in 50 mM acetic acid. Trypsin was stored at -80˚C. Human Butyrylcholinesterase was purified from outdated human plasma as described (Lockridge et al., 2005). Purified human transferrin, human complement component 3, human alpha-2-macroglubin, and human alpha-1anti-trypsin were purchased from Sigma.

Human plasma labeled with FP-biotin. 2 to 10 ml of human plasma was treated with 200 µM FP-biotin for 5 h at room temperature. Unreacted FP-biotin was removed by dialysis. A second preparation of FP-biotin labeled plasma was depleted of albumin by chromatography on a column of Affi-gel blue. A third preparation of FP-biotinylated plasma was depleted of the 12 most abundant plasma proteins by binding to antibodies in the Proteome Partitioning Kit.

Purification of FP-biotin-labeled proteins. FP-biotin-labeled proteins were purified by binding to tetrameric avidin beads, where binding is so tight that the proteins can only be released by boiling in SDS. Preparations of this type were loaded on SDS polyacrylamide gels for separation of individual FP-biotinylated proteins. FP-biotin-labeled proteins were also purified on monomeric avidin beads from which they were released with 10% acetic acid.

Purification of FP-biotin-labeled peptides. In some experiments the FP-

biotin-labeled peptides rather than FP-biotin-labeled proteins were purified on monomeric avidin beads where the peptides were released with 10% acetic acid.

Digestion with trypsin or pepsin. FP-biotin labeled proteins were denatured in 8 M urea, disulfide bonds were reduced with 10 mM dithiothreitol at pH 8.0, and carbamidomethylated with 0.1 M iodoacetamide. Samples were desalted by dialysis against 10 mM ammonium bicarbonate pH 8.5. The equivalent of 200 µl plasma, containing 10 mg of protein, was digested with 0.1 mg of trypsin in 20 mM ammonium bicarbonate pH 8.5 at 37˚C for 4 to 16 h. Trypsin was inactivated with 10 mM DFP before the sample was loaded on monomeric avidin beads.

For digestion with pepsin, the pH of the plasma sample was reduced to pH 2.1 with 1% trifluoroacetic acid. 10 mg plasma protein was digested with 0.05 mg pepsin for 2 h at 37˚C. The pepsin was inactivated by raising the pH to 8.9 before loading the digest on monomeric avidin beads.

Controls. The negative control was human plasma treated with all reagents except FP-biotin. The positive control was FP-biotinylated human Butyrylcholinesterase.

Gel electrophoresis. FP-biotinylated plasma proteins were separated on SDS polyacrylamide gels or on nondenaturing polyacrylamide gels. Gels were stained with Coomassie blue or with Microwave Blue. Gel bands were cut out and prepared for digestion with trypsin as described (Peeples et al., 2005).

Protein blot. Proteins were transferred from a polyacrylamide gel to PVDF membrane and hybridized with Streptavidin Alexafluor 680 (Molecular Probes). Fluorescence intensity was captured in the Odyssey Infrared Imaging System (LiCor). This procedure visualized FP-biotinylated proteins.

MALDI-TOF mass spectrometry. All peptide samples were screened by MALDI-TOF before they were analyzed in the Q-Trap mass spectrometer, because MALDI-TOF is a quick way to estimate the number of FP-biotinylated peptides and their masses. Salt-free peptides were plated on a stainless steel target plate in 1 µl aliquots, allowed to dry, and overlaid with 10 mg/ml CHCA matrix in 50% acetonitrile, 0.1% trifluoroacetic acid. Mass spectra were acquired with the Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA).

Tandem quadrupole mass spectrometry at UNMC. Tandem mass spectra (MS/MS) were acquired on a Q-Trap 2000 triple quadrupole linear ion trap mass spectrometer (Applied Biosystems) with a nano electrospray ionization source. Mass spectra were calibrated using fragment ions generated from collision-induced dissociation of Glu fibrinopeptide B (Sigma). Enhanced product ion scans were obtained with collision energy of 40±5 V and nitrogen gas pressure of 4×10^{-5} Torr.

Thermo-Finnigan LTQ linear ion trap mass spectrometry at Wistar. One set of FP-biotin labeled human plasma proteins was sent to Dr. Kaye D. Speicher at the Wistar Proteomics Facility in Philadelphia, PA for identification. The FP-biotinylated proteins had been purified on tetrameric avidin beads and separated by SDS gel electrophoresis. The same set of proteins was also analyzed at UNMC on our Q-Trap mass spectrometer.

Data analysis. Hundreds of extracted ion chromatograms were manually searched for the presence of the characteristic fragment ions of FP-biotin. Ion masses of 227, 312, 329, and 591 amu in the extracted ion chromatogram indicate that the peptide carries the FP-biotin tag on serine or threonine (Schopfer et al., 2005). Ion masses of 227, 312, and 329 in the absence of mass 591 indicate FP-biotin is covalently bound to tyrosine.

Peptides that fragmented to give ions characteristic of FP-biotin were manually sequenced from data in the extracted ion chromatogram.

Results

Mass spectrometry of FP-biotinylated human plasma proteins. Analysis of FP-biotin labeled proteins extracted from gel slices has tentatively identified the 31 proteins listed in Table 1.1. What is tentative is the interpretation that these proteins bind OP. Until we actually identify the residue that binds the OP or perform a protein blot with purified protein, we consider the identification tentative. We are certain that the 9 proteins in Table 1.2 bind OP covalently. The 4 serine hydrolases - plasminogen, prothrombin, complement component 1 s subcomponent, and butyrylcholinesterase - bind OP on the active site serine. Five additional proteins - albumin, transferrin, complement component 3, alpha-2-macroglobulin, and anti-trypsin - bind FP-biotin covalently. The evidence for this conclusion comes from protein blots and from mass spectrometry. When the purified proteins were treated with FP-biotin, boiled in SDS, subjected to SDS gel electrophoresis, and transferred to PVDF membrane, the blots gave an intense fluorescent signal with Streptavidin Alexafluor 680. This demonstrated that human albumin, human transferrin, human complement component 3, human alpha-2-macroglobulin, and human anti-trypsin covalently bind FP-biotin.

Tyr 411 of albumin. Mass spectrometry was used to identify the amino acid to which the FP-biotin was covalently attached. Tyrosine 411 of human albumin is the attachment site for FP-biotin. Tyrosine 411 is also the covalent binding site for soman, sarin, DFP, chlorpyrifos oxon and dichlorvos. Our manuscript on this subject has been accepted for publication and is attached. The tryptic peptide containing the active site tyrosine has the sequence (Arg)TyrThrLys. This peptide is located near the surface of the albumin molecule (Ghuman et al., 2005).

Table 1.1. Preliminary identification of OP binding proteins in human plasma.

	protein	MOWSE	# peptides	MW	gi #	GXSXG
1	hemoglobin chain D	366	9	15869	1431652	-
2	hemoglobin chain C	111	3	15389	493852	-
3	serum albumin	451	13	69366	28592	-
4	inter-alpha globulin inhibitorH2	349	10	105217	55958063	-
5	inter-alpha globulin inhibitorH1	254	6	101403	4504781	-
6	alpha-2 macroglobulin	1985	51	163175	25303946	-
7	ceruloplasmin	117	4	115473	1620909	-
8	complement component 5	334	13	141723	179692	-
9	plasminogen	279	8	90496	190026	GDSGG
10	IG GI HNie	295	7	49174	229601	-
11	immunoglobulin kappa light chain VLJ region	365	7	29280	21669423	GGSGG
12	fibrinogen gamma	211	7	49450	182439	-
13	gelsonin isoform b	304	9	80641	38044288	-
14	alpha fibrinogen precursor	264	4	69767	182424	-
15	chain E, fragment double-D from fibrin	189	7	37649	28373962	-
16	fibrinogen alpha A	283	5	49398	223918	-
17	complement component 3	409	11	187046	4557385	-
18	IgG kappa chain	332	6	23419	4176418	-
19	IgM kappa IIIb SON	329	5	13765	224377	-
20	proapolipoprotein	158	4	28962	178775	-
21	fibronectin 1 isoform 6 preproprotein	3250	31	243078	47132549	GGSRG GNSNG GNSNG GLSPG
22	fibronectin homolog	470	14	272269	31873670	GGSRG GNSNG GNSNG GLSPG GNSLG
23	prothrombin	1052	10	70038	4503635	GDSGG
24	lipoprotein B100	5013	114	512880	225311	-
25	complement component C1s	677	18	76685	41393602	GDSGG
26	butyrylcholinesterase	712	18	68419	4557351	GESAG
27	transferrin	391	20	77051	15021381	GRSAG
28	Ig mu chain C region	119	9	49557	127514	-
29	Igg B12	275	10	50417	15825648	-
30	IGHA1 protein	275	7	53,377	21619010	-
31	alpha-1 antitrypsin	227	15	46,709	54695780	-

MOWSE score is the probability of a match between the experimental data and the peptide mass in the database. MOWSE scores greater than 69 are significant ($p<0.05$). # peptides is the number of peptides whose sequence matched the protein. MW is the molecular weight. gi # is the accession number. GXSXG is the consensus sequence around the active site serine.

Table 1.2. Human plasma proteins that bind FP-biotin covalently.

protein	gi #	OP binding site
albumin	28592	Tyr 411
transferrin	15021381	Tyr 238
alpha-2-macroglobulin	163175	
complement component 3	4557385	
alpha-1 anti-trypsin	54695780	
plasminogen	190026	Ser 760
prothrombin	4503635	Ser 568
complement component C1s	41393602	Ser 195
butyrylcholinesterase	4557351	Ser 198

The OP binding site is not yet known for 3 proteins in Table 2. However, we are confident that these 3 proteins bind OP covalently because protein blots of FP-biotinylated pure proteins give a strong signal when hybridized with Streptavidin Alexafluor 680.

Tyr 238 of transferrin. Tryptic peptides of pure human transferrin labeled with FP-biotin were isolated by binding to monomeric avidin beads. Bound peptides were eluted with 10% acetic acid and analyzed in the Q-Trap mass spectrometer by LC/MS/MS. A search of the enhanced product ion spectra has revealed several FP-biotinylated peptides. One of these has been sequenced to date. This has resulted in the identification of Tyrosine 238 as the OP binding site of human transferrin. The sequence of the OP-peptide is KPVDEYK where OP is bound to Y.

Other OP-tyrosine peptides. FP-biotinylated human plasma proteins were digested with trypsin or pepsin, isolated on monomeric avidin beads, and analyzed by LC/MS/MS. Many peptides were found to contain the characteristic product ions of FP-biotin. The majority of these had ions 227, 312, and 329 but did not have ion 591. This meant that FP-biotin was bound to tyrosine rather than to serine or threonine (Schopfer et al., 2005). The signals from two peptides were strong enough to allow one to deduce the amino acid sequence. The sequences were YPR and AYPR. In both peptides FP-biotin was covalently bound to Y. These sequences were too short to allow assignment to a parent protein.

UNMC and Wistar. Proteins in a set of 6 gel slices were analyzed at UNMC on our Q-Trap mass spectrometer and at Wistar on their LTQ-Thermo Finnigan mass spectrometer. The gel slices were from the same FP-biotinylated plasma preparation run on the same polyacrylamide gel. The results from both institutes were essentially the same, thus reassuring us that our techniques and equipment were satisfactory.

Discussion

Serine hydrolases are not the only OP binding proteins in human plasma. Our expectation when we started this project was that OP-labeled proteins would all be serine esterases and serine proteases. We expected butyrylcholinesterase to be the dominant OP-binding protein in human plasma (Fidder et al., 2002; Van Der Schans et al., 2004). Our results show that this expectation was not met. We have found that OP bind not only to serine esterases and serine proteases, but also to proteins that have an activated tyrosine. We have conclusively demonstrated that albumin and transferrin bind OP on tyrosine. We expect to identify many more proteins that bind OP on tyrosine.

Our hypothesis is that each OP binds to a set of proteins, and that the sets do not overlap completely. For example, almost all OP bind to acetylcholinesterase and butyrylcholinesterase, but not all OP bind to albumin. We have been unable to demonstrate binding of VX or iso-OMPA to albumin. The identity of the proteins bound by a particular OP depends on the affinity of that particular OP, and therefore on the precise chemical structure of that OP. The OP literature has mostly studied nerve agents and DFP, thus limiting the view of the type of proteins capable of binding OP.

Motif for OP binding to tyrosine. We have positively identified Tyrosine 411 of albumin and Tyrosine 238 of transferrin as sites that covalently bind OP in human plasma. In addition we have identified two tryptic peptides where OP was bound to Tyrosine. In each case the tyrosine was near an arginine or lysine. The arginine or lysine is likely involved in activating the tyrosine, lowering its pKa value and allowing the hydroxyl group to ionize at physiologic pH. We propose that a common motif for OP-reactive tyrosines may be the presence of a nearby arginine or lysine.

Relevance. An understanding of the set of proteins modified by exposure to a particular OP is expected to help in understanding why some people become chronically ill from a low dose exposure that has no effect on the average individual. A sensitive person may have a mutation in an OP-binding protein that reduces the person's tolerance to a low dose.

Knowledge of the set of proteins modified by OP exposure is also going to be useful for diagnosis of OP exposure. Proteins that have a longer half-life in the circulation can be tested weeks after exposure. Mass spectrometry assays rely on the ability of peptides to ionize in the electrospray; some OP-modified peptides may ionize more readily than others. Confirmation of exposure may be more certain if a set of proteins is proven to have been modified rather than a single protein.

The results of our work are expected to lead to an antibody based assay for OP exposure. The OP binding site in albumin is located on the surface of the albumin molecule, where the OP and the surrounding amino acids make an excellent epitope. A dipstick type assay is envisioned using antibody to OP-albumin.

Task 1

Task 1. Identify proteins labeled by FP-biotin
1.3. Identify FP-biotin labeled proteins in mouse blood
1.4. Identify the amino acid covalently attached to FP-biotin in each FP-biotinylated mouse protein.

Relation to statement of work. Results for tasks 1.3 and 1.4 are reported.

Tasks 1.3 and 1.4

OP-labeled proteins in mouse plasma

Summary
The goal was to identify proteins in mouse plasma that bind FP-biotin. Mass spectrometry identified three carboxylesterases in mouse plasma, the major carboxylesterase being ES1. In addition albumin, two forms of hemoglobin, serine protease inhibitor, contraspin, and plakoglobin were identified.

Introduction

The reason for duplicating our efforts in mouse and human plasma is that the mouse will be used for in vivo studies. Once we know the proteins to look for, we will inject mice with various doses of FP-biotin and quantify the amount of each labeled protein by mass spectrometry. This will indicate which proteins are likely to be important in FP-biotin toxicity.

It is known that mouse plasma differs from human plasma in that mouse plasma is rich in carboxylesterase but human plasma has no carboxylesterase (Maxwell et al., 1987; Li et al., 2005).

Materials and Methods

The materials and methods are the same as described for Tasks 1.1 and 1.2. An 80 µl aliquot of mouse plasma from strain 129Sv mice was depleted of albumin by chromatography on Affi-gel blue, and labeled with FP-biotin. The FP-biotinylated proteins were purified by binding to immobilized avidin beads. The proteins were released from the beads by boiling in SDS gel loading buffer, and separated on an SDS gel. Coomassie stained bands were cut out. Each band

was treated with dithiothreitol to reduce protein disulfide bonds, followed by alkylation with iodoacetamide. Proteins were digested with trypsin and the tryptic peptides were extracted from the gel. The peptides were subjected to LC/MS/MS analysis on the Q-Trap mass spectrometer. Proteins were identified by analysis of fragmentation patterns using Mascot software.

Results and Discussion

Table 1.3 lists the FP-biotinylated proteins identified in mouse plasma.

Table 1.3 FP-biotin binding proteins in mouse plasma (strain 129Sv).

protein	MOWSE	# peptides	MW	Gi #	OP binding site
ES1 carboxylesterase	845	18	61266	22135640	GESSG
carboxylesterase precursor	782	16	61140	2921308	GESSG
liver carboxylesterase N precursor, lung surfactant convertase isoform 3	508	10	60782	82918153	GSSAG
albumin	506	10	68693	29612571	RYTQK
hemoglobin alpha	157	3	15085	49900	-
hemoglobin beta	130	3	15840	1183932	-
serine protease inhibitor	117	2	46866	15079234	-
contraspin	97	3	46673	54173	-
contraspin	96	2	46965	50442	-
plakoglobin	37	2	68151	423532	-

MOWSE score is the probability of a match between the experimental data and the peptide mass in the database. MOWSE scores greater than 69 are significant ($p<0.05$). # peptides is the number of peptides whose sequence matched the protein. MW is the molecular weight. Gi# is the accession number. The active site serine for OP binding is S in the sequence GXSXG. The active site tyrosine for OP binding is Y.

Carboxylesterases. Mouse plasma was analyzed on the Q-Trap three times. Three carboxylesterases were identified. The major carboxylesterase was ES1. A good score for ES1 was found in all 3 experiments. The carboxylesterase precursor was found in 2 experiments, while the liver carboxylesterase was found only in one experiment, after the sample had been concentrated prior to analysis on the mass spectrometer. This suggests that the carboxylesterase precursor and the liver carboxylesterase have a lower abundance than ES1 in mouse plasma.

In a previous mass spectrometry study we also found ES1, but no other carboxylesterase, suggesting that ES1 is indeed the most abundant carboxylesterase in mouse plasma (Peeples et al., 2005). Others have also found ES1 (gi: 22135640) in mouse plasma by LC/MS/MS (Bhat et al., 2005).

Biochemical characterization of the carboxylesterases in mouse serum classified ES1 as the principal carboxylesterase in mouse serum (Otto et al., 1981).

The question of which carboxylesterases are present in mouse plasma is relevant to the effort to make a carboxylesterase knockout mouse. Mice have 16 carboxylesterase genes located in two clusters on chromosome 8 (Mus musculus chromosome 8, reference assembly; NC 000074). From our mass spectrometry results it can be expected that knockout of carboxylesterase ES1 will substantially reduce the carboxylesterase activity of mouse blood but will not eliminate it completely.

Albumin. Mouse plasma was depleted of albumin prior to labeling and mass analysis, because protein blots had revealed that albumin bound most of the FP-biotin. Albumin scavenged 1000 more molecules of FP-biotin than were scavenged by butyrylcholinesterase (Peeples et al., 2005) in a living mouse. Despite the depletion step, there was enough albumin left in the 2.4 mg of processed protein to show up as an OP-labeled tryptic peptide in the mass spectrometer. The mouse albumin peptide that binds OP has the sequence RYTQK with FP-biotin bound to Y.

Other OP-labeled proteins. We are not convinced that hemoglobin, contraspin, and plakoglobin are labeled with OP. These proteins could have bound to avidin beads nonspecifically. We will require additional proof before we have a conclusive answer. The additional proof is of two types. 1) Protein blots of purified proteins treated with FP-biotin must hybridize with Streptavidin Alexafluor 680. This type of experiment conclusively proves that a protein covalently binds FP-biotin, but it does not identify the site of binding. 2) The peptide containing the FP-biotin label must ionize in the mass spectrometer and must fragment to allow its amino acid sequence to be deduced from the fragmentation pattern. Evidence of this type provides irrefutable proof.

Significance. The OP-binding proteins in mouse plasma are expected to parallel the OP-binding proteins in human plasma, with the major exception of carboxylesterase. Human plasma contains no carboxylesterase, whereas mouse plasma is rich in carboxylesterase (Li et al., 2005). It is important to know the identity of the OP-binding proteins in mouse plasma because the mouse will be used for in vivo studies. In the future we will inject mice with various OP and use mass spectrometry to identify and quantify the proteins that have been modified by each OP.

TASK 2

Task 2. Identify proteins labeled by DFP
2.2. Label pure human albumin with DFP, isolate the DFP-labeled tryptic peptide, and identify the characteristic ion fragments produced by collision induced dissociation.

Relation to statement of work. Results for task 2.2 are reported.

Task 2.2

DFP-labeled human albumin digested with trypsin and fragmented by collision induced dissociation

Summary
Our goal was to determine whether diisopropylfluorophosphate (DFP) covalently binds to human albumin. A second goal was to determine the fragmentation pattern of the DFP-albumin tryptic peptide. Human albumin was treated with DFP at alkaline pH, digested with pepsin at pH 2.3, and analyzed by MADLI-TOF mass spectrometry. Two singly charged peaks, 1718 and 1831 m/z, corresponding to the unlabeled peptide fragments containing the active site Tyr 411 residue, were detected in all samples. The sequences of the two peptides were VRYTKKVPQVSTPTL and LVRYTKKVPQVSTPTL. The DFP adducts of these peptides had masses of 1881 and 1994; these masses fit a mechanism whereby DFP bound covalently to Tyr 411. The binding of DFP to Tyr 411 of human albumin was confirmed by electrospray tandem mass spectrometry and analysis of product ions. The fragmentation pattern yielded characteristic ions that will be useful for selected ion monitoring of DFP exposure. The OP-albumin adduct did not lose an alkoxy group, leading to the conclusion that aging did not occur. The presence of Tyr 411 on an exposed surface of albumin suggests that an antibody response could be generated against OP-albumin adducts.

Introduction

The acute toxicity of organophosphorus toxicants (OP) is known to be due to inhibition of acetylcholinesterase. However, other proteins also bind OP though their role in toxicity is less defined (Casida and Quistad, 2004). Albumin is a

potential new biomarker of OP exposure. Mice treated with a nontoxic dose of a biotinylated nerve agent analog, FP-biotin (10-fluoroethoxyphosphinyl-N-biotinamidopentyldecanamide), had 1000 times more FP-biotinylated albumin than FP-biotinylated butyrylcholinesterase in their blood (Peeples et al., 2005).

Albumin has been shown to covalently bind radiolabeled diisopropylfluorophosphate (DFP). Human albumin incorporated 1 mole of DFP per mole of albumin when 20-70 μM albumin was incubated with a 7-fold molar excess of DFP at pH 8.0 for 2 h at 23˚C (Means and Wu, 1979; Hagag et al., 1983). Bovine albumin also incorporated 1 mole of DFP per mole of albumin (Murachi, 1963). The site of covalent binding of DFP to human albumin was identified by amino acid sequencing. The labeled peptide had the sequence ArgTyrThrLys with DFP bound to Tyr (Sanger, 1963). Later, when the complete amino acid sequence of human albumin was known, the active site tyrosine was identified as Tyr 411 (Tyr 435 when residue #1 is Met of the signal peptide). Mass spectrometry identified Tyr 410 of bovine albumin (equivalent to Tyr 411 of human albumin) as the covalent binding site for FP-biotin (Schopfer et al., 2005). The nerve agents soman and sarin were shown to bind covalently to human albumin on tyrosine (Black et al., 1999; Adams et al., 2004) and to be released by treatment with potassium fluoride (Adams et al., 2004).

Albumin has also been demonstrated to be an OP hydrolase, hydrolyzing chlorpyrifos oxon, O-hexyl O-2, 5-dichlorophenylphosphoramidate, and paraoxon at measurable rates (Erdos and Boggs, 1961; Ortigoza-Ferado et al., 1984; Sultatos et al., 1984; Sogorb et al., 1998a). The apparent Km of bovine albumin is 0.41 mM for chlorpyrifos oxon, 1.85 mM for paraoxon (Sultatos et al., 1984) and that of human albumin is 3.6 mM for DFP (Means and Wu, 1979). Despite this seemingly consistent body of results, some issues have been raised regarding the reaction of OP with albumin. It has been questioned whether the observed OP hydrolase activity was associated with the albumin molecule itself, or with minor phosphotriesterase contaminants in the albumin preparation (Erdos and Boggs, 1961). In addition, the possibility has been raised that DFP binds to one site in albumin, but that other OP bind to a different site (Mourik and de Jong, 1978; Sultatos et al., 1984).

We developed a MALDI-TOF assay for identifying DFP-labeled human albumin. In addition we determined the fragmentation pattern for the DFP-labeled human albumin tryptic peptide. Knowledge of the fragmentation pattern will allow development of a highly sensitive selective ion monitoring assay to test for exposure to DFP.

Materials and Methods

Materials. Purified human serum albumin, essentially fatty acid free (Fluka via Sigma, St. Louis, MO; cat no. 05418), pepsin (Sigma, St. Louis, MO; cat no. P6887 from porcine gastric mucosa), modified trypsin, sequencing grade (Promega, Madison, WI; cat no. V5113), diisopropylfluorophosphate (Sigma; cat no. D0879), acetonitrile (HPLC grade 99% ACROS cat no. 61001-0040 from

Fisher Scientific, Pittsburgh, PA), trifluoroacetic acid, sequencing grade (Beckman Instruments, Palo Alto, CA; cat no. 290203), 2,5-dihydroxybenzoic acid (DHBA) matrix (Applied Biosystems Foster City, CA), alpha-cyano 4-hydroxycinnamic acid (CHCA) (Sigma; cat no. 70990) was recrystallized before use. Calibration standards for MALDI-TOF were from New England Biolabs (Beverly, MA; cat no. P7720S). They included Angiotensin 1, 1297.51 amu, ACTH (7-38) 3660.19 amu, and ACTH (18-39) 2466.73 amu. Double distilled water was prepared in-house and autoclaved.

Preparation of DFP-albumin peptic peptides. Fatty acid-free human albumin at a concentration of 10 mg/ml, which is 150 µM, was dissolved in 25 mM ammonium bicarbonate pH 8.6 and treated with an equimolar concentration of DFP for 24 h at 37˚C. The pH of 1000 µl reaction mixture was reduced to pH 2.3 by the addition of 500 µl of 1% trifluoroacetic acid. Pepsin was dissolved in 10 mM HCl to make 1 mg/ml and stored at -80˚C. The albumin was digested with pepsin (1:250 ratio) for 2 hours at 37˚C and diluted to 1 pmol/µl with 0.1% trifluoroacetic acid.

MALDI-TOF. A 1 µl aliquot of diluted peptic digest was applied to a stainless steel target plate, air dried, and overlaid with 1 µl of 2,5-dihydroxybenzoic acid matrix. The CHCA matrix gave similar results. Mass spectra were acquired with the Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA) in linear positive ion mode, 20,000 volts accelerating voltage, 94% grid voltage, 0.1% guide wire, 350 nsec extraction delay time, automated laser intensity adjustment from 1000 to 1600. The instrument was calibrated with a peptide calibration mixture from New England Biolabs. Mass accuracy for each standard was within 0.05% of the corresponding average molecular weight. Spectra were acquired in automatic mode, by examining signals from random spots on a target. The signals from the first 10 spots that met the acceptance criteria were summed into one final profile mass spectrum The acceptance criteria were signal intensities between 1000 and 55000 counts, with signal to noise ratios of 10 or greater, and minimum resolution of 50. The final spectrum was the average of 1000 shots.

The MS-Digest program from the UCSF Mass Spectrometry Facility was used to calculate the masses of the peptic peptides expected from digests of human serum albumin.

Quadrupole mass spectrometer. MS/MS spectra were acquired on a Q-Trap 2000 triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA) with a nano electrospray ionization source. DFP-labeled albumin digested with trypsin was infused into the mass spectrometer via a fused silica emitter (360 µm o.d., 20 µm i.d., 10 µm taper from New Objective, Woburn, MA), using a Harvard syringe pump to drive a 100 µl Hamilton syringe equipped with an inline 0.25 micron filter, at a flow rate of 1 µl/min. Samples were prepared in 50% acetonitrile, 0.1% formic acid. Positive ion spectra were obtained. Mass spectra were calibrated using fragment ions generated from

collision-induced dissociation of Glu fibrinopeptide B (Sigma). Enhanced product ion scans were obtained with a collision energy of 40±5 volts and a nitrogen gas pressure of 4 x 10^{-5} Torr. The final enhanced product ion scan was the average of 212 scans.

Results

Reaction of pure human albumin with DFP. Fatty acid free-albumin was used because fatty acids and DFP bind to the same albumin domain and therefore fatty acids could block the binding of OP (Means and Wu, 1979; Peters, 1996). The covalent attachment site for human albumin, Tyr 411, is located near the surface of the albumin molecule where it is accessible to proteases. Digestion with trypsin at pH 8.6 or with pepsin at pH 2-2.5 released peptides of the expected masses without the need to denature or to reduce and alkylate the disulfide bonds of albumin. Peptides containing Tyr 411 had the sequence YTK (411 m/z, singly charged mass) when the protease was trypsin, and the sequences VRYTKKVPQVSTPTL (1718 m/z) and LVRYTKKVPQVSTPTL (1831 m/z) when the protease was pepsin. Pepsin routinely missed one cleavage in our experiments.

The tryptic YTK peptide (411 m/z) and the DFP adduct had masses that overlapped with matrix peaks, which made them difficult to detect by MALDI mass spectrometry. Furthermore, the YTK peptide and YTK-DFP adduct did not seem to ionize when irradiated by the nitrogen laser in the Voyager DE-PRO, though they did ionize in the electrospray source of the Q-Trap. By contrast, the larger peptides produced by digestion of albumin with pepsin separated well from matrix and ionized to give good signals in the Voyager DE-PRO. Therefore, samples intended for analysis by MALDI-TOF were digested with pepsin rather than with trypsin.

Figure 2.1 shows the MALDI-TOF spectra obtained for pepsin-digested human albumin before and after treatment with DFP. The top panel shows masses at 1718 and 1831 m/z, which are consistent with the peptides from unlabeled albumin that contain Tyr 411. Additional albumin peptides are also present but they do not contain Tyr 411 and are therefore not discussed. The DFP panel shows two new peaks at 1882 and 1995 m/z for the diisopropoxyphosphate adducts. About 70% of the albumin is labeled. Since the MALDI conditions disrupt non-covalent interactions, the DFP-peptide adducts must be covalently formed. These results support the conclusion that human albumin is labeled by DFP and are consistent with the site for covalent attachment being Tyr 411. The masses correspond to the dialkoxy adducts rather than the monoalkoxy adducts. Masses for monoalkoxy adducts were not found, supporting the conclusion that the DFP-albumin adduct does not age.

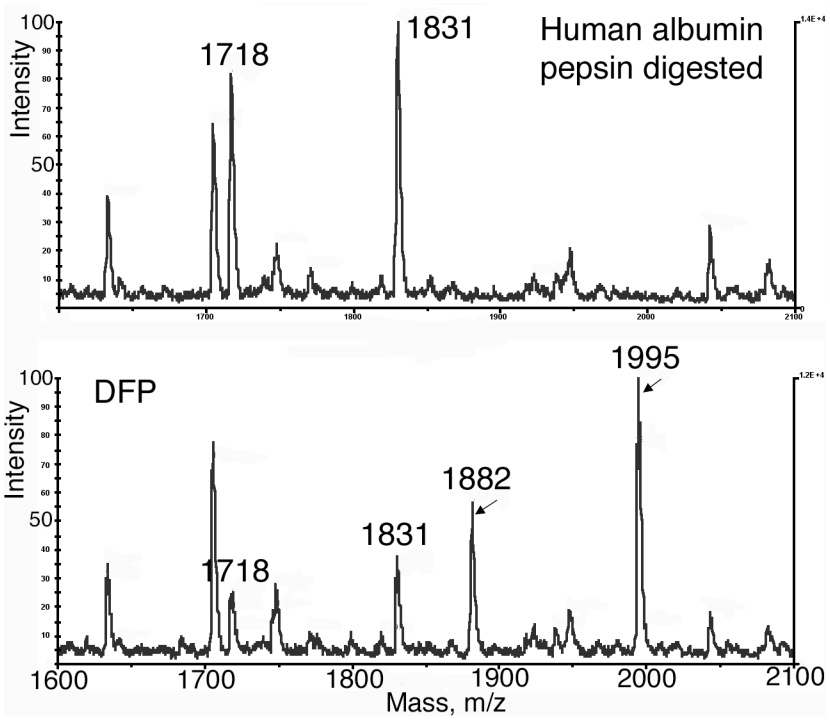

Figure 2.1. MALDI-TOF analysis of human albumin-DFP adducts. (Top panel) Digestion of human albumin with pepsin at pH 2.3 yields 2 peptides containing Tyr 411 whose average mass to charge ratios are 1718 and1831 (singly protonated). (DFP panel) Incubation of human albumin with DFP yields diisopropoxyphosphate adducts of 1882 and 1995 m/z. Samples were diluted to 1 pmol/µl before plating 1 µl on the MALDI target with 2,5-dihydroxybenzoic acid matrix.

Saturating the albumin binding sites. Unlabeled 1718 and 1831 m/z peptides were always present when the concentration of DFP was the same as the concentration of albumin. However, when the DFP concentration was 40-fold higher than the albumin concentration, all the Tyr 411 sites were occupied and no unlabeled peptides of 1718 and 1831 m/z were detected. DFP labeling reactions with albumin were performed at pH 8.5 because labeling occurs at high pH but is markedly decreased at neutral pH (Murachi, 1963; Means and Wu, 1979).

Limit of detection. It was essential to dilute the albumin and plasma digests before applying the sample on the target plate. Undiluted samples did not show the desired peptides due to ion suppression and charge competition effects. The limit of detection of DFP-labeled peptide was determined from dilutions of peptides in which all of the Tyr 411 sites were occupied. The diluent was 0.1% trifluoroacetic acid in water. Peaks of interest were detected after 100, 1000,

3000, and 9,000 fold dilution of a sample whose starting concentration was 600 μM (40 mg/ml) albumin. The signal to noise ratio for the 1:9000 diluted sample was 3:1. At 18,000 fold dilution the peak height was only two fold above the noise. Thus, the minimum detectable signal from a DFP-labeled peptide occurred at 0.07 pmol/μl.

MS/MS analysis confirms DFP binding to Tyr 411 of the YTK peptide. DFP-labeled human albumin was digested with trypsin and the digest was infused into the Q-Trap mass spectrometer. The enhanced mass spectrum showed a peak at 575.4 m/z, which is consistent with the singly charged YTK peptide covalently bound to DFP ($[M+H]^{+1}$ = 411 amu for the YTK peptide plus 164 amu added mass from DFP). This peptide was subjected to collision-induced dissociation with nitrogen as the collision gas. The resulting enhanced product ion spectrum yielded amino acid sequence information consistent with the sequence YTK where DFP is covalently bound to tyrosine. See Figure 2.2. A mass was found at 147.2 amu, indicative of the C-terminal lysine from a y-series. This was followed by masses at 248.3, for the ThrLys dipeptide, and at 575.4, for the Tyr*ThrLys tripeptide, including the N-terminus, from a y-series (where Tyr* represents the diisopropylphospho-adduct of tyrosine). No relevant signals were found at higher mass. No evidence for the diisopropylphospho-adduct of threonine was found.

Further, convincing evidence for covalent binding of DFP to Tyr 411 comes from the presence of 6 masses that are all consistent with various fragments of DFP attached to tyrosine, alone or in conjunction with the YTK peptide. The structures of these 6 ions are shown in Figure 2.2. As mentioned before, the ion at 575.4 amu is consistent with the singly protonated YTK peptide plus the added mass from covalent attachment of DFP. Neutral loss of 42 amu yields the 533.4 amu ion. Loss of 42 amu is predicted for b-elimination of propylene from the diisopropylphosphate adduct. This b-elimination type reaction, also referred to as a McLafferty rearrangement (McLafferty, 1959; Fredriksson et al., 1995) is a facile reaction commonly seen during collision induced dissociation of phosphopeptides (McLachlin and Chait, 2001). A second neutral loss of 42 amu yields the 491.3 amu ion; this mass is consistent with a phosphorylated YTK peptide. In theory, all three of these masses are consistent with DFP adducts of either tyrosine or threonine. However, the mass at 244.2 amu is characteristic of an N-terminal phosphotyrosine, b-series aziridone ion, and the 226.2 amu ion is consistent with its dehydration product (Mann et al., 2001). In addition, the mass at 216.2 amu is characteristic of a phosphotyrosine immonium ion (Steen et al., 2001). Furthermore, no indication of phosphorylated or organophosphorylated threonine was found. These results prove that DFP covalently binds to Tyr 411 of human albumin.

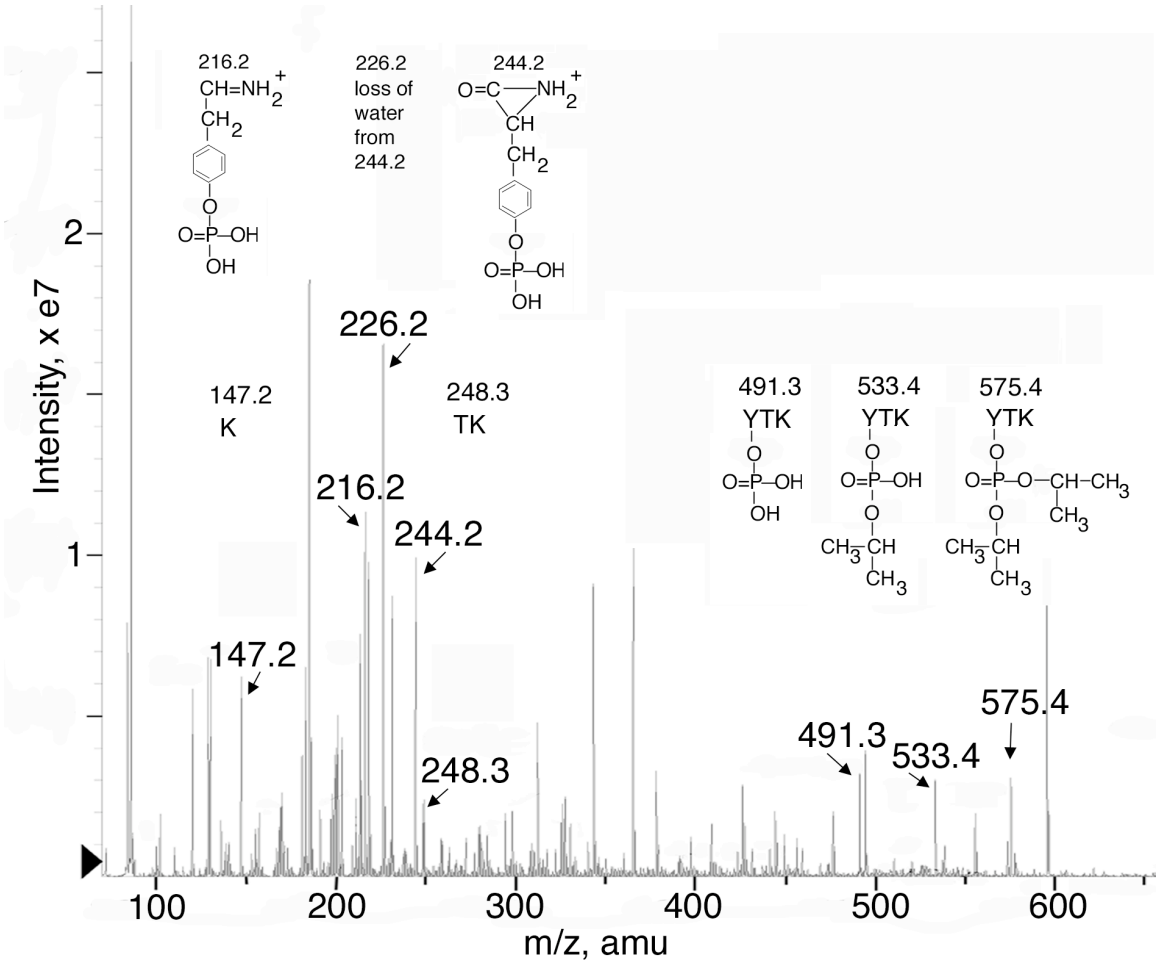

Figure 2.2. Product ion spectrum of DFP-labeled human albumin peptide of mass 575.4. DFP-labeled albumin was digested with trypsin and infused into the Q-Trap mass spectrometer. The singly charged parent ion of 575.4 m/z has the sequence YTK and has the diisopropylphosphate group covalently bound to tyrosine. Six fragment masses and their corresponding structures are shown.

No evidence for any form of the dephosphorylated YTK peptide was found. This probably reflects the relative difficulty of releasing OP from tyrosine, compared to the other fragmentation pathways available to the diisopropylphospho-YTK peptide. Facile dephosphorylation of phospho-tyrosine via a beta-elimination type mechanism is not available because tyrosine does not afford a suitable environment. Beta-elimination would require the shift of a proton from the beta carbon of the leaving group to the phosphate oxygen, with concomitant formation of a double bond between the alpha and beta carbons of the leaving group. For phospho-tyrosine, the beta-proton of the leaving tyrosine is aromatic and therefore not readily released; furthermore, formation of the double bond requires introduction of a triple bond into the aromatic ring of

tyrosine, another difficult operation. Though phosphate can be released from tyrosine during collision induced dissociation, it seems to require the presence of hydroxyl moieties on the phosphate. Even then, the yield of dephosphorylation is poor (Fredriksson et al., 1995; Tholey et al., 1999). Thus, it would appear that elimination of propylene from the diisopropoxy-moieties and fragmentation of the peptide backbone provide energetically more acceptable routes for utilization of the collision energy than dephosphorylation of the tyrosine.

The presence of two isopropyl groups in parent ion 575.4 supports the conclusion that the DFP-albumin adduct does not age.

The unique set of 6 phosphorylated fragment ions in Figure 2.2 for DFP-albumin could be useful for identifying exposure to DFP in a mass spectrometry method that selectively searches for characteristic fragment ions.

Only one covalent binding site for OP. Human albumin labeled with DFP and digested with pepsin was searched for additional peptides that might bind OP. MALDI-TOF and Q-Trap analysis revealed no other DFP adducts. The only identified OP binding site was Tyr 411.

Discussion

Mechanism of OP labeling of albumin. DFP was found to label Tyr 411 of human albumin. The stoichiometry of labeling has been shown to be one mole ^{3}H-DFP or ^{14}C-DFP incorporated per mole of albumin (Murachi, 1963; Means and Wu, 1979; Hagag et al., 1983; Sultatos et al., 1984). The specific labeling of one tyrosine in a molecule that contains 18 tyrosines suggests that Tyr 411 is in a special environment. This tyrosine has an unusually low pKa of 7.9 - 8.3 (Means and Wu, 1979; Ahmed et al., 2005), in contrast to the pKa of 10 for the average tyrosine.

Tyr 411 is the active site residue not only for reaction with OP, but also for reaction with esters such as p-nitrophenyl acetate, carbamates such as carbaryl, and amides such as o-nitroacetanilide (Means and Wu, 1979; Sogorb et al., 1998b; Sogorb et al., 2004; Manoharan and Boopathy, 2006). The esterase and amidase activity of albumin can be inhibited by pretreatment with DFP. Conversely, labeling with DFP can be prevented by pretreatment with p-nitrophenyl acetate, which forms a stable acylated albumin adduct (Means and Wu, 1979). The sensitivity of albumin esterase activity to ionic strength led Means and Wu to conclude that the reactive tyrosine residue is located on the surface of albumin in an apolar environment adjacent to several positively charged groups. This description of the OP binding site of albumin was proven to be correct when the crystal structure was solved (He and Carter, 1992; Sugio et al., 1999). Subdomain IIIa of albumin contains a pocket lined by hydrophobic side chains. The hydroxyl of Tyr 411 is close to the side chains of Arg 410 and Lys 414.

Site-directed mutagenesis experiments have shown that albumin esterase activity is abolished when Tyr 411 is mutated to Ala, and severely diminished

when Arg 410 is mutated to Ala (Watanabe et al., 2000). These results support Tyr 411 as the active site for albumin esterase activity and support a role for Arg 410 in stabilizing the reactive anionic form of Tyr 411. The negatively charged Tyr 411 is available for nucleophilic attack on ester and amide substrates. Though crystal structures of several ligand albumin complexes have been solved (Ghuman et al., 2005), the crystal structure of an OP-albumin adduct is not yet available.

No aging of OP-albumin adducts. Aging of OP-labeled acetylcholinesterase and butyrylcholinesterase is defined as the loss of an alkoxy group from the OP-labeled active site serine (Benschop and Keijer, 1966; Michel et al., 1967). The nerve agents sarin, soman, and VX yield the same aged OP derivative, so that these agents may be difficult to distinguish when bound to acetylcholinesterase or butyrylcholinesterase (Millard et al., 1999a; Millard et al., 1999b).

The covalent bond between DFP and the active site serine of acetylcholinesterase and butyrylcholinesterase undergoes aging. However DFP bound to albumin does not age. Aging is a catalytic process that requires the participation of nearby histidine and glutamic acid residues (Kovach et al., 1997). Residues that promote aging are not present in the active site pocket of albumin. The absence of aging allows the bound OP to be spontaneously released from Tyr 411. This makes albumin an OP hydrolase, though a very slow one. Albumin hydrolyzes O-hexyl O-2,5-dichlorophenylphosphoramidate, chlorpyrifos oxon, and paraoxon (Erdos and Boggs, 1961; Ortigoza-Ferado et al., 1984; Sultatos et al., 1984; Sogorb et al., 1998a).

Antibody to OP-albumin. Our results suggest the possibility of detecting OP exposure through use of an antibody detection assay directed toward the OP-albumin adduct at Tyr 411. There is precedent for the generation of antibodies to very small haptens bound to protein. For example, antibodies that distinguish between phosphotyrosine, phosphoserine, and phosphothreonine have been successfully produced (Glenney et al., 1988; Levine et al., 1989). Antibodies to soman, sarin, and VX bound to carrier proteins through a chemical linker have been produced (Grognet et al., 1993; Zhou et al., 1995; Johnson et al., 2005) . The proposed OP-albumin epitope could be more useful for detection of OP exposure than existing antibodies because the OP-albumin adduct has no chemical linker and no foreign protein environment.

An OP-albumin adduct at Tyr 411 may generate an antibody response in exposed individuals and the antibody could be detected to determine a history of exposure to OP. This would facilitate monitoring exposure to OP long after the exposure incident and long after the antigen has disappeared.

A copy of a publication that reports these results is attached.

Bin Li, Lawrence M. Schopfer, Steven H. Hinrichs, Patrick Masson, Oksana Lockridge (2007) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay for organophosphorus toxicants bound to human albumin at Tyr 411. Analytical Biochemistry. In press.

KEY RESEARCH ACCOMPLISHMENTS

- Two new biomarkers of exposure to OP have been identified in human blood: albumin and transferrin.
- Both albumin and transferrin bind OP covalently to tyrosine. This finding is contrary to the expectation that OP bind exclusively to serine.
- A new mass spectrometry assay has been developed to measure OP exposure in blood. The assay uses MALDI-TOF to measure the mass of the albumin active site peptide after covalent OP binding. This identifies the fact of OP exposure and also identifies the OP.

REPORTABLE OUTCOMES

- Manuscript accepted for publication. A copy is attached.

Bin Li, Lawrence M. Schopfer, Steven H. Hinrichs, Patrick Masson, Oksana Lockridge (2007) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay for organophosphorus toxicants bound to human albumin at Tyr 411. Analytical Biochemistry. In press.

CONCLUSION

Our mass spectrometry results contradict the dogma that serine esterases and serine proteases are the only class of proteins modified by exposure to OP. We have found that tyrosine 411 of human albumin and tyrosine 238 of human transferrin are covalently modified by OP. The significance of this finding is in diagnosis of OP exposure. It now becomes possible to look for several proteins in human blood, in addition to butyrylcholinesterase and acetylcholinesterase, for evidence of OP exposure. The method we have developed to date for diagnosis of OP exposure is a mass spectrometry method. However, it now becomes possible to develop a simple antibody based assay to use in the field. The information we obtained from mass spectrometry allows us to propose a natural epitope on the surface of human albumin as the OP antigen. A dipstick type assay for OP exposure is planned as the ultimate result of this work.

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay for organophosphorus toxicants bound to human albumin at Tyr411

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Abstract

Our goal was to determine whether chlorpyrifos oxon, dichlorvos, diisopropylfluorophosphate (DFP), and sarin covalently bind to human albumin. Human albumin or plasma was treated with organophosphorus (OP) agent at alkaline pH, digested with pepsin at pH 2.3, and analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The singly charged peaks *m*/*z* 1718 and 1831, corresponding to the unlabeled peptide fragments containing the active site Tyr411 residue, were detected in all samples. The sequences of the two peptides were VRYTKKVPQVSTPTL and LVRYTKKVPQVSTPTL. The peptide–OP adducts of these peptides were also found. They had masses of 1854 and 1967 for chlorpyrifos oxon, 1825 and 1938 for dichlorvos, 1881 and 1994 for DFP, and 1838 and 1938 for sarin; these masses fit a mechanism whereby OP bound covalently to Tyr411. The binding of DFP to Tyr411 of human albumin was confirmed by electrospray tandem mass spectrometry and analysis of product ions. None of the OP–albumin adducts lost an alkoxy group, leading to the conclusion that aging did not occur. Our results show that OP pesticides and nerve agents bind covalently to human albumin at Tyr411. The presence of Tyr411 on an exposed surface of albumin suggests that an antibody response could be generated against OP–albumin adducts.

Keywords: Biomarker organophosphate exposure; Pepsin; Sarin; Soman; Dichlorvos; Diisopropylfluorophosphate; Chlorpyrifos oxon; Nerve agents; Pesticides

The acute toxicity of organophosphorus (OP)[1] toxicants is known to be due to inhibition of acetylcholinesterase. However, other proteins also bind OP, although their role in toxicity is less defined [1]. Albumin is a potential new biomarker of OP exposure. Mice treated with a nontoxic dose of a biotinylated nerve agent analog, FP-biotin (10-fluoroethoxyphosphinyl-*N*-biotinamidopentyldecanamide), had 1000 times more FP-biotinylated albumin than FP-biotinylated butyrylcholinesterase in their blood [2].

Albumin has been shown to covalently bind radiolabeled diisopropylfluorophosphate (DFP). Human albumin incorporated 1 mol DFP per mole of albumin when 20 to 70 μM albumin was incubated with a sevenfold molar excess of DFP at pH 8.0 for 2 h at 23 °C [3,4]. Bovine albumin also incorporated 1 mol DFP per mole of albumin [5]. The site of covalent binding of DFP to human albumin was identified by amino acid sequencing. The labeled peptide

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[1] *Abbreviations used:* OP, organophosphorus; FP-biotin, 10-fluoroethoxyphosphinyl-*N*-biotinamidopentyldecanamide; DFP, diisopropylfluorophosphate; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; DHBA, 2,5-dihydroxybenzoic acid; CHCA, α-cyano 4-hydoxycinnamic acid; ACTH, adrenocorticotropic hormone; MS/MS, tandem mass spectra; LC–MS, liquid chromatography–mass spectrometry; GC–MS, gas chromatography–mass spectrometry.

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had the sequence ArgTyrThrLys with DFP bound to Tyr [6]. Later, when the complete amino acid sequence of human albumin was known, the active site tyrosine was identified as Tyr411 (Tyr435 when residue 1 is Met of the signal peptide). Mass spectrometry (MS) identified Tyr410 of bovine albumin (equivalent to Tyr411 of human albumin) as the covalent binding site for FP-biotin [7]. The nerve agents soman and sarin were shown to bind covalently to human albumin on tyrosine [8,9] and to be released by treatment with potassium fluoride [9].

Albumin has also been demonstrated to be an OP hydrolase, hydrolyzing chlorpyrifos oxon, *O*-hexyl *O*-2,5-dichlorophenylphosphoramidate, and paraoxon at measurable rates [10–13]. The apparent K_m of bovine albumin is 0.41 mM for chlorpyrifos oxon and 1.85 mM for paraoxon [12], and the apparent K_m of human albumin is 3.6 mM for DFP [3]. Despite this seemingly consistent body of results, some issues have been raised regarding the reaction of OP with albumin. It has been questioned whether the observed OP hydrolase activity was associated with the albumin molecule itself or with minor phosphotriesterase contaminants in the albumin preparation [10]. In addition, the possibility has been raised that DFP binds to one site in albumin but that other OP toxicants bind to a different site [12,14].

Our goal was to determine whether Tyr411 of human albumin was the site for covalent attachment of a variety of OP toxicants. For this purpose we developed a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS assay applicable to purified human albumin and to human plasma.

Materials and methods

Materials

Purified human serum albumin, essentially fatty acid free, was obtained from Fluka via Sigma (Cat. No. 05418, St. Louis, MO, USA). Pepsin from porcine gastric mucosa was obtained from Sigma (Cat. No. P6887). Modified trypsin, sequencing grade, was purchased from Promega (Cat. No. V5113, Madison, WI, USA). DFP was obtained from Sigma (Cat. No. D0879). Dichlorvos and chlorpyrifos oxon were purchased from Chem Services (Cat Nos. PS-89 and MET-674B, West Chester, PA, USA). Sarin-treated human plasma was a gift from Patrick Masson. Acetonitrile high-performance liquid chromatography (HPLC)-grade 99% ACROS, was purchased from Fisher Scientific (Cat. No. 61001-0040, Pittsburgh, PA, USA). Trifluoroacetic acid (TFA), sequencing grade, was purchased from Beckman Instruments (Cat. No. 290203, Palo Alto, CA, USA). 2,5-Dihydroxybenzoic acid (DHBA) matrix was purchased from Applied Biosystems (Foster City, CA, USA). α-Cyano 4-hydroxycinnamic acid (CHCA, Cat. No. 70990, Sigma) was recrystallized before use. Calibration standards for MALDI-TOF were obtained from New England Biolabs (Cat. No. P7720S, Beverly, MA, USA) and included angiotensin 1 (1297.51 amu), adrenocorticotropic hormone (ACTH) (7–38) (3660.19 amu), and ACTH (18–39) (2466.73 amu). Double distilled water was prepared in-house and was autoclaved.

Sample preparation for DFP-, dichlorvos-, and chlorpyrifos oxon-treated samples

Fatty acid-free human albumin at a concentration of 10 mg/ml, which is 150 μM, was dissolved in 25 mM ammonium bicarbonate (pH 8.6) and treated with an equimolar concentration of OP for 24 h at 37 °C. The pH of 1000 μl reaction mixture was reduced to 2.3 by the addition of 500 μl of 1% TFA. Pepsin was dissolved in 10 mM HCl to make 1 mg/ml and was stored at −80 °C. The albumin was digested with pepsin (1:250 ratio) for 2 h at 37 °C and was diluted to 1 pmol/μl with 0.1% TFA.

A 200-μl aliquot of human plasma was treated with 6.85 μl of 20 mM OP (660 μM final OP concentration) for 24 h at 37 °C. The pH was adjusted to 2.3 to 2.5 by the addition of 200-μl of 1% TFA. Proteins were digested with 50 μl of 1 mg/ml pepsin for 2 h at 37 °C. Before spotting the digest on the target plate, a 10-μl aliquot of the digest was diluted with 390 μl of 0.1% TFA so that the final plasma dilution was 1000-fold.

MALDI-TOF

A 1-μl aliquot of diluted peptic digest was applied to a stainless-steel target plate, air-dried, and overlaid with 1 μl of 2,5-dihydroxybenzoic acid matrix. The CHCA matrix gave similar results. Mass spectra were acquired with the Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems/MDS Sciex) in linear positive ion mode, 20,000 V accelerating voltage, 94% grid voltage, 0.1% guide wire, 350 ns extraction delay time, and automated laser intensity adjustment from 1000 to 1600. The instrument was calibrated with a peptide calibration mixture from New England Biolabs. Mass accuracy for each standard was within 0.05% of the corresponding average molecular weight. Spectra were acquired in automatic mode by examining signals from random spots on a target. The signals from the first 10 spots that met the acceptance criteria were summed into one final profile mass spectrum The acceptance criteria were signal intensities between 1000 and 55,000 counts with signal/noise ratios of 10 or greater and minimum resolution of 50. The final spectrum was the average of 1000 shots.

The MS-Digest program from the UCSF Mass Spectrometry Facility was used to calculate the masses of the peptic peptides expected from digests of human serum albumin.

Quadrupole mass spectrometer

Tandem mass spectra (MS/MS) were acquired on a Q-Trap 2000 triple quadrupole linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex) with a nano electrospray

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ionization source. DFP-labeled albumin digested with trypsin was infused into the mass spectrometer via a fused silica emitter (360 μm o.d., 20 μm i.d., 10 μm taper, New Objective, Woburn, MA, USA) using a Harvard syringe pump to drive a 100-μl Hamilton syringe equipped with an inline 0.25-μ filter at a flow rate of 1 μl/min. Samples were prepared in 50% acetonitrile and 0.1% formic acid. Positive ion spectra were obtained. Mass spectra were calibrated using fragment ions generated from collision-induced dissociation of Glu fibrinopeptide B (Sigma). Enhanced product ion scans were obtained with collision energy of 40 ± 5 V and nitrogen gas pressure of 4×10^{-5} Torr. The final enhanced product ion scan was the average of 212 scans.

Sample preparation of sarin-treated plasma

Human plasma (100 μl) was treated with 600 μM sarin and stored at ambient temperature for 3 days. This concentration of sarin is equimolar with the concentration of albumin in plasma. Then 10 μl was digested with 0.5 μg of pepsin at 37 °C for 2 h at pH 2.3, and the peptides were separated by HPLC on a Waters 625 LC system. A C18 reverse-phase column (Prodigy 5-μ ODS(2), 100 × 4.6 mm, 5 μ, 00 D-3300-E0, Phenomenex, Torrance, CA, USA) was used to trap the peptides from the digest, which were then eluted with a 40-min gradient starting with 85% buffer A (0.1% TFA in water) and 15% buffer B (acetonitrile containing 0.07% TFA) and ending with 65% buffer A and 35% buffer B. Then 1-ml fractions were reduced in volume to 200 μl in a vacuum centrifuge, and 1 μl was analyzed by MALDI-TOF. A control plasma sample was treated identically except that it was incubated with 3.5% isopropanol rather than with sarin.

Results

Reaction of pure human albumin with OP

The assay was developed with pure human albumin and later tested with human plasma. Fatty acid-free albumin was used because fatty acids and OP bind to the same albumin domain and therefore fatty acids could block the binding of OP [3,15]. The covalent attachment site for human albumin, Tyr411, is located near the surface of the albumin molecule, where it is accessible to proteases. Digestion with trypsin at pH 8.6 or with pepsin at pH 2.0 to 2.5 released peptides of the expected masses without the need to denature or to reduce and alkylate the disulfide bonds of albumin. Peptides containing Tyr411 had the sequence YTK (*m*/*z* 411, singly charged mass) when the protease was trypsin and had the sequences VRYTKKVPQVSTPTL (*m*/*z* 1718) and LVRYTKKVPQVSTPTL (*m*/*z* 1831) when the protease was pepsin. Pepsin routinely missed one cleavage in our experiments.

The tryptic YTK peptide (*m*/*z* 411) and the dichlorvos, chlorpyrifos oxon, and DFP adducts had masses that overlapped with matrix peaks, making them difficult to detect by MALDI–MS. Furthermore, the YTK peptide and YTK–OP adducts did not seem to ionize when irradiated by the nitrogen laser in the Voyager DE-PRO, although they did ionize in the electrospray source of the Q-Trap mass spectrometer. In contrast, the larger peptides produced by digestion of albumin with pepsin separated well from matrix and ionized to give good signals in the Voyager DE-PRO. Therefore, samples intended for analysis by MALDI-TOF were digested with pepsin rather than with trypsin.

Table 1 lists the expected peptic peptide masses before and after covalent binding of dichlorvos, chlorpyrifos oxon, DFP, and sarin to Tyr411 of human albumin. The leaving group in Table 1 is that portion of the OP molecule that detaches from the OP on covalent binding of the OP to protein. The mass of the leaving group is absent from the final adduct. The added OP mass comes from the phosphorus atom, the two phosphorus ligands, and the phosphoryl oxygen atom, less one hydrogen.

Fig. 1 shows the MALDI-TOF spectra obtained for pepsin-digested human albumin before and after treatment with OP. The top panel shows masses at *m*/*z* 1718 and 1831, which are consistent with the peptides from unlabeled albumin that contain Tyr411. Additional albumin peptides are also present, but they do not contain Tyr411 and therefore are not discussed. The dichlorvos panel shows peaks at *m*/*z* 1718 and 1831 as well as two new peaks at *m*/*z* 1826 and 1939. The two new peaks have the expected sizes for the dimethoxyphosphate adducts of the *m*/*z* 1718 and 1831 peptides. The amount of labeled albumin estimated from the relative peak areas is 65%. The chlorpyrifos oxon panel shows two new peaks at *m*/*z* 1854 and 1967 for the diethoxyphosphate adducts. Approximately 30% of the albumin is labeled. The DFP panel shows two new peaks at *m*/*z* 1882 and 1995 for the diisopropoxyphosphate adducts. Approximately 70% of the albumin is labeled. Because the MALDI conditions disrupt noncovalent interactions, the OP–peptide adducts must be covalently formed. These results support the conclusion that human albumin is labeled by dichlorvos, chlorpyrifos oxon, and DFP and are consistent with the site for covalent attachment being Tyr411. The masses correspond to the dialkoxy adducts rather than to the monoalkoxy adducts. Masses for monoalkoxy adducts were not found, supporting the conclusion that OP–albumin adducts do not age.

Saturating the albumin binding sites

Unlabeled *m*/*z* 1718 and 1831 peptides always were present when the concentration of OP was the same as the concentration of albumin. However, when the dichlorvos or DFP concentration was 40-fold higher than the albumin concentration, all of the Tyr411 sites were occupied and no unlabeled peptides of *m*/*z* 1718 and 1831 were detected. OP labeling reactions with albumin were performed at pH 8.5 because labeling occurs at high pH but is decreased markedly at neutral pH [3,5].

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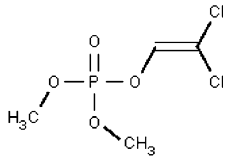
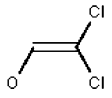
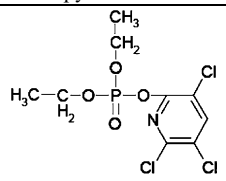
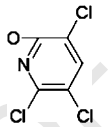
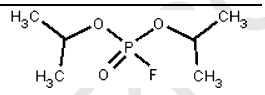
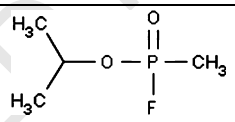
Table 1
Pepsin digested human albumin

Human albumin peptide	Peptide *m/z*	Peptide *m/z* after dichlorvos	Dichlorvos	Leaving group	Added mass
VRYTKKVPQVSTPTL	1718	1826			108
LVRYTKKVPQVSTPTL	1831	1939			
		Peptide *m/z* after chlorpyrifos oxon	Chlorpyrifos oxon		
VRYTKKVPQVSTPTL	1718	1854			136
LVRYTKKVPQVSTPTL	1831	1967			
		Peptide *m/z* after DFP	DFP		
VRYTKKVPQVSTPTL	1718	1882		F	164
LVRYTKKVPQVSTPTL	1831	1995			
		Peptide *m/z* after sarin	Sarin		
VRYTKKVPQVSTPTL	1718	1838		F	120
LVRYTKKVPQVSTPTL	1831	1951			

Note. The $[M+H]^{+1}$ masses of peptic peptides containing Tyr411 are listed before and after covalent binding of OP. Tyr411 is underlined. The accession number for human albumin is GI:28592 where Tyr411 is Tyr435 because numbering begins with the signal peptide.

Limit of detection

It was essential to dilute the albumin and plasma digests before applying the sample on the target plate. Undiluted samples did not show the desired peptides due to ion suppression and charge competition effects. The limit of detection of OP-labeled peptide was determined from dilutions of peptides in which all of the Tyr411 sites were occupied. The diluent was 0.1% TFA in water. Peaks of interest were detected after 100-, 1000-, 3000-, and 9000-fold dilutions of a sample whose starting concentration was 600 μM (40 mg/ml) albumin. The signal/noise ratio for the 1:9000 diluted sample was 3:1. At 18,000-fold dilution, the peak height was only twofold above the noise. Thus, the minimum detectable signal from an OP-labeled peptide occurred at 0.07 pmol/μl.

Missed cleavage

We hoped to increase the sensitivity of the assay by changing digestion conditions so that pepsin would consistently cleave Leu from the N terminus of the active site peptide. If there were no missed cleavage, the area of the 1721 peak would increase relative to the background noise, thereby increasing the sensitivity of the assay. We increased the pepsin/albumin ratio to 1:25, lowered the pH in increments to 1.5, and increased the digestion time to 4 h. A pepsin/albumin ratio of 1:25 at pH 1.8 incubated for 2.5 h at 37 °C did eliminate the 1831 peptide but did not increase the signal for 1721.

Detection of dichlorvos bound to albumin in human plasma

The concentration of albumin in human plasma is approximately 40 to 50 mg/ml (600–770 μM). No other protein in plasma is present at such a high concentration. This overwhelming concentration of albumin in plasma suggested that it might be possible to detect OP–albumin adducts without separating albumin from other proteins in plasma. This was tested by incubating an aliquot of human plasma with a concentration of dichlorvos equimolar to the albumin concentration in plasma. The pH was not adjusted, and no buffer was added for the reaction with dichlorvos. Then the plasma was digested with pepsin at pH 2.3 and diluted 600-fold with 0.1% TFA to yield an albumin peptide concentration of approximately 1 pmol/μl. A 1-μl aliquot of the diluted digest was applied to the MALDI target. The control sample was human plasma treated in parallel with everything except dichlorvos. Fig. 2 shows that albumin peptides at *m/z* 1718 and 1831 stand out, despite the presence of normal plasma components, and that the OP-labeled form of these peptides at *m/z* 1826 and

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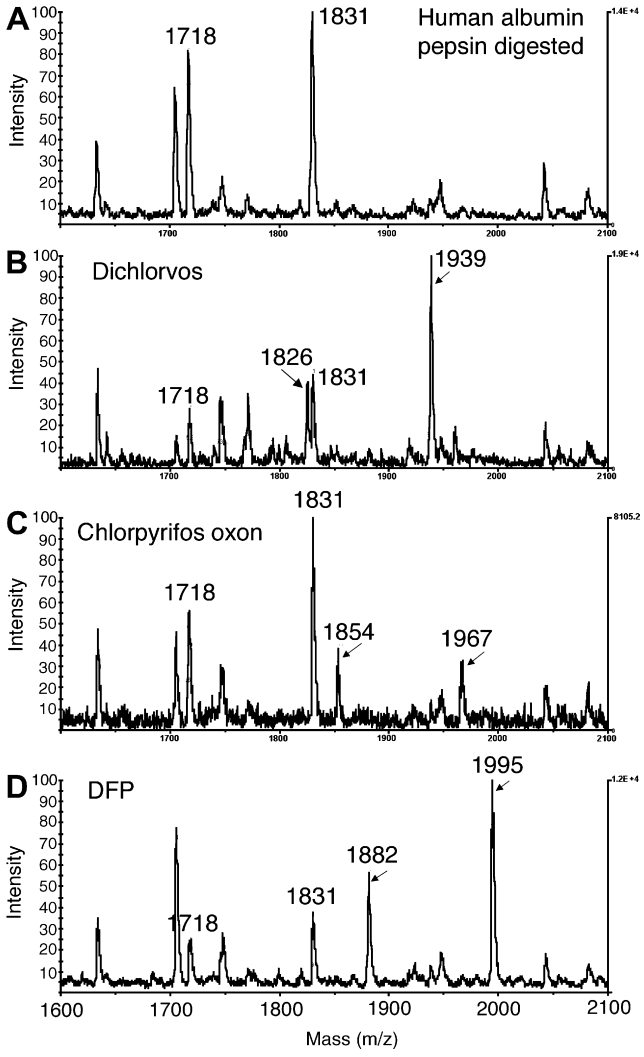

Fig. 1. MALDI-TOF analysis of human albumin–OP adducts. (A) Digestion of human albumin with pepsin at pH 2.3 yields two peptides containing Tyr411 whose average mass/charge ratios are 1718 and 1831 (singly protonated). (B) Incubation of human albumin with dichlorvos in ammonium bicarbonate (pH 8.5), followed by digestion with pepsin (pH 2.3), yields dimethoxyphosphate adducts of *m/z* 1826 and 1939. (C) Incubation with chlorpyrifos oxon yields diethoxyphosphate adducts of *m/z* 1854 and 1967. (D) Incubation with DFP yields diisopropoxyphosphate adducts of *m/z* 1882 and 1995. Samples were diluted to 1 pmol/μl before plating 1 μl on the MALDI target with 2,5-dihydroxybenzoic acid matrix.

1939 can be detected by MALDI–TOF. We conclude that human plasma can be assayed for OP bound to albumin. The 1826 and 1939 masses are 108 amu larger than their unlabeled counterparts, identifying the OP as a dimethoxy OP and thus classifying it as a pesticide rather than as a nerve agent. For forensic purposes, it is valuable to know whether the OP is a pesticide or a nerve agent.

Detection of sarin bound to albumin in human plasma

MALDI-TOF analysis of peptic digests of human plasma labeled by reaction with 600 μM sarin did not show the sarin-labeled peptides after simple dilution. We suspected that the absence of signal was due to ion suppression. Therefore, the digests were fractionated by reverse-phase chromatography prior to mass spectral analysis, as shown in Fig. 3. Fractions were collected at 1-min intervals. Each HPLC fraction was analyzed by MALDI-TOF. Peptides of interest eluted between 8 and 16 min. The unlabeled active site peptides of albumin, of masses 1718 and 1831, eluted at 8 to 10 min. The sarin-labeled peptides of masses 1838 and 1951 eluted at 14 to 16 min (Fig. 4, fractions 14 and 16). The peptides of interest separated from a large peak of UV absorbing material in the HPLC. Elimination of this material would be expected to reduce ion suppression, thereby accounting for the appearance of the sarin-labeled peptide signals in MALDI-TOF analysis.

Fig. 4 shows the MALDI-TOF spectra for the two sarin-labeled peptides of human albumin with masses of 1838 and 1951. These masses are consistent with the 1718 and 1831 albumin peptides to which 120 amu from sarin has been added. The fact that the sarin–peptide complex survived the MALDI conditions indicates that sarin has made a covalent complex. These results show that sarin covalently binds to human albumin on Tyr411. They also show that binding can be detected in plasma and that the sarin adduct of albumin does not lose an alkoxy group during storage for 3 days at room temperature.

Confirmation of DFP binding to Tyr411 of the YTK peptide

DFP-labeled human albumin was digested with trypsin, and the digest was infused into the Q-Trap mass spectrometer. The enhanced mass spectrum showed a peak at *m/z* 575.4, which is consistent with the singly charged YTK peptide covalently bound to DFP ($[M+H]^{+1} = 411$ amu for the YTK peptide plus 164 amu added mass from DFP [Table 1]). This peptide was subjected to collision-induced dissociation with nitrogen as the collision gas. The resulting enhanced product ion spectrum yielded amino acid sequence information consistent with the sequence YTK where DFP is covalently bound to tyrosine (Fig. 5). A mass was found at 147.2 amu, indicative of the C-terminal lysine from a *y*-series. This was followed by masses at 248.3, for the ThrLys dipeptide, and at 575.4, for the Tyr*ThrLys tripeptide, including the N terminus, from a *y*-series (where Tyr* represents the diisopropylphospho adduct of tyrosine). No relevant signals were found at higher masses. No evidence for the diisopropylphospho adduct of threonine was found.

Furthermore, convincing evidence for covalent binding of DFP to Tyr411 comes from the presence of six masses, all of which are consistent with various fragments of DFP attached to tyrosine alone or in conjunction with the YTK peptide. The structures of these six ions are shown in Fig. 5. As mentioned earlier, the ion at 575.4 amu is consistent with the singly protonated YTK peptide plus the added mass from covalent attachment of DFP. Neutral loss of 42 amu yields the 533.4-amu ion. Loss of 42 amu is predicted for β-elimination of propylene from the diisopropylphosphate adduct. This β-elimination-type reaction, also referred to as a McLafferty rearrangement [16,17], is a facile

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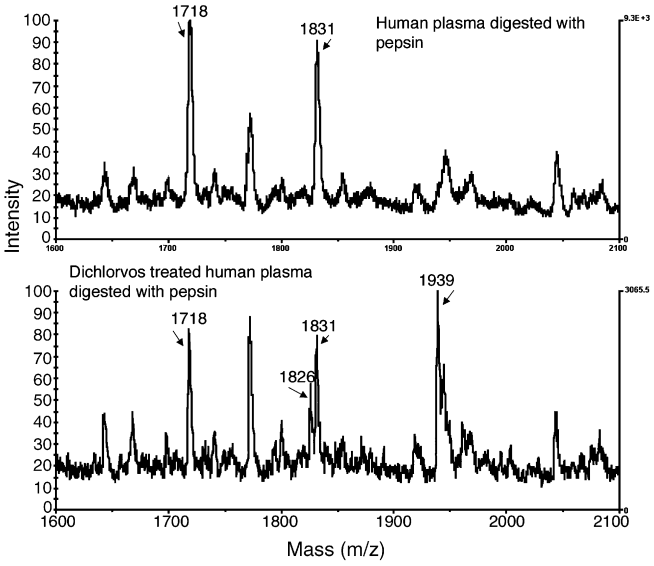

Fig. 2. MALDI-TOF analysis of OP–albumin adducts in human plasma. The top panel is the control sample. Human plasma digested with pepsin shows the Tyr411 containing albumin peptides of *m/z* 1718 and 1831. The bottom panel shows two new peaks at *m/z* 1826 and 1939 in dichlorvos-treated human plasma, representing peptides containing dichlorvos covalently bound to Tyr411 of albumin.

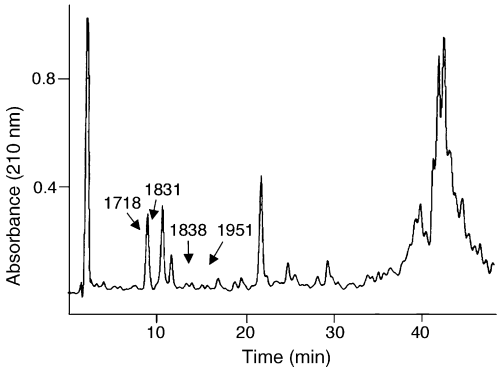

Fig. 3. HPLC trace. A peptic digest of human plasma (10 μl) that had been treated with sarin was subjected to reverse-phase chromatography. Unlabeled active site albumin peptides of masses 1718 and 1831 separated from sarin-labeled albumin peptides of masses 1838 and 1951 and from a large peak of UV absorbing material. The marked peaks contain a mixture of peptides; therefore, the relative UV intensities do not represent the relative amounts of labeled and unlabeled albumin.

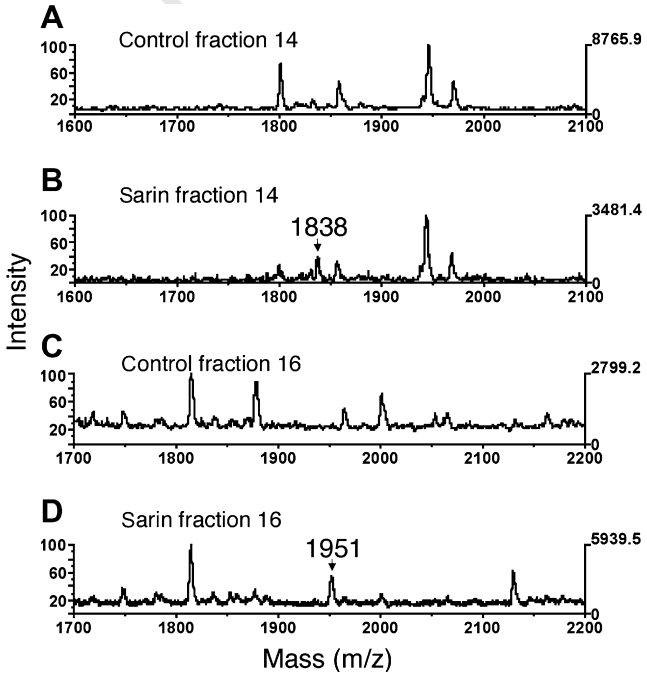

Fig. 4. MALDI-TOF analysis of sarin covalently attached to human albumin. (A and C) Control human plasma digested with pepsin and fractionated by HPLC but not treated with sarin. (B and D) Human plasma treated with sarin before digestion with pepsin and fractionation by HPLC. Fractions were collected at 1-min intervals. Sarin-labeled albumin peptides of 1838 and 1951 amu include 120 amu from sarin.

reaction commonly seen during collision-induced dissociation of phosphopeptides [18]. A second neutral loss of 42 amu yields the 491.3-amu ion; this mass is consistent with a phosphorylated YTK peptide. In theory, all three of these masses are consistent with DFP adducts of either tyrosine or threonine. However, the mass at 244.2 amu is characteristic of an N-terminal phosphotyrosine, *b*-series aziridone ion, and the 226.2-amu ion is consistent with its dehydration product [19]. In addition, the mass at 216.2 amu is characteristic of a phosphotyrosine immonium ion [20]. Furthermore, no indication of phosphorylated or organophosphorylated threonine was found. These results prove that DFP covalently binds to Tyr411 of human albumin.

No evidence for any form of the dephosphorylated YTK peptide was found. This probably reflects the relative difficulty of releasing OP from tyrosine compared with the other fragmentation pathways available to the diisopropylphospho–YTK peptide. Facile dephosphorylation of phosphotyrosine via a β-elimination-type mechanism is not available because tyrosine does not afford a suitable environment. β-Elimination would require the shift of a proton from the β-carbon of the leaving group to the phosphate oxygen, with concomitant formation of a double bond

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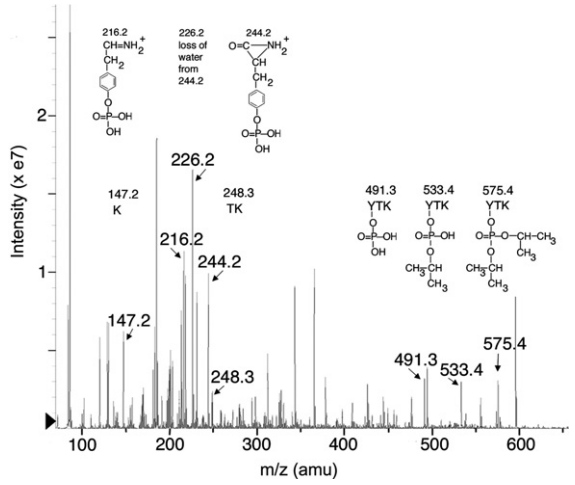

Fig. 5. Product ion spectrum of DFP-labeled human albumin peptide 575.4. DFP-labeled albumin was digested with trypsin and infused into the Q-Trap mass spectrometer. The singly charged parent ion of *m*/*z* 575.4 has the sequence YTK and has the diisopropylphosphate group covalently bound to tyrosine. Fragment masses and their corresponding structures are shown. In addition to information on the DFP–YTK peptide, fragments from a second albumin peptide (LVNEVTEFAK, doubly protonated) can be extracted from the fragmentation information.

between the α- and β-carbons of the leaving group. For phosphotyrosine, the β-proton of the leaving tyrosine is aromatic and therefore not readily released; furthermore, formation of the double bond requires introduction of a triple bond into the aromatic ring of tyrosine—another difficult operation. Although phosphate can be released from tyrosine during collision-induced dissociation, it seems to require the presence of hydroxyl moieties on the phosphate. Even then, the yield of dephosphorylation is poor [17,21]. Thus, it would appear that elimination of propylene from the diisopropoxy moieties and fragmentation of the peptide backbone provide energetically more acceptable routes for use of the collision energy than does dephosphorylation of the tyrosine.

The spectrum in Fig. 5 contains a number of major peaks that were not used in the analysis of the DFP–YTK adduct. Most of these are attributable to fragments from a second albumin peptide. The doubly charged form of LVNEVTEFAK appears at *m*/*z* 575. The peaks at 218, 365, 494, and 595 amu (plus additional peaks at the higher masses of 694, 823, 937, and 1036 amu) correspond to the complete *y*-series for this contaminating peptide. Peaks at 213, 327, 456, and 555 amu can be assigned to a *b*-series from this same peptide, whereas peaks at 312, 343, 426, and 444 amu reflect internal fragments. The large peak at 86 amu is the immonium ion of Leu/Ile.

The presence of two isopropyl groups in parent ion 575.4 supports the conclusion that the DFP–albumin adduct does not age.

The unique set of six phosphorylated fragment ions in Fig. 5 for DFP–albumin could be useful for identifying exposure to DFP in an MS method that selectively searches for characteristic fragment ions.

Only one covalent binding site for OP

Human albumin labeled with dichlorvos or DFP and digested with pepsin was searched for additional peptides that might bind OP. MALDI-TOF and Q-Trap analysis revealed no other OP adducts. The only identified OP binding site was Tyr411.

Discussion

Mechanism of OP labeling of albumin

Each of the OP toxicants tested in this work labeled Tyr411 of human albumin. The stoichiometry of labeling has been shown to be 1 mol ^{3}H[DFP] or ^{14}C[DFP] incorporated per mole of albumin [3–5,12]. The specific labeling of 1 tyrosine in a molecule that contains 18 tyrosines suggests that Tyr411 is in a special environment. This tyrosine has an unusually low pK_a of 7.9 to 8.3 [3,22], in contrast to the pKa of 10 for the average tyrosine.

Tyr411 is the active site residue not only for reaction with OP but also for reaction with esters such as *p*-nitrophenyl acetate, carbamates such as carbaryl, and amides such as *o*-nitroacetanilide [3,23–25]. The esterase and amidase activity of albumin can be inhibited by pretreatment with DFP. Conversely, labeling with DFP can be prevented by pretreatment with *p*-nitrophenyl acetate, which forms a stable acylated albumin adduct [3]. The sensitivity of albumin esterase activity to ionic strength led Means and Wu to conclude that the reactive tyrosine residue is located on the surface of albumin in an apolar environment adjacent to several positively charged groups [3]. This description of the OP binding site of albumin was proven to be correct when the crystal structure was solved [26,27]. Subdomain IIIa of albumin contains a pocket lined by hydrophobic side chains. The hydroxyl of Tyr411 is close to the side chains of Arg410 and Lys414.

Site-directed mutagenesis experiments have shown that albumin esterase activity is abolished when Tyr411 is mutated to Ala and is severely diminished when Arg410 is mutated to Ala [28]. These results support Tyr411 as the active site for albumin esterase activity and support a role for Arg410 in stabilizing the reactive anionic form of Tyr411. The negatively charged Tyr411 is available for nucleophilic attack on ester and amide substrates. Although crystal structures of several ligand albumin complexes have been solved [29], the crystal structure of an OP–albumin adduct is not yet available.

No aging of OP–albumin adducts

Aging of OP-labeled acetylcholinesterase and butyrylcholinesterase is defined as the loss of an alkoxy group from the OP-labeled active site serine [30,31]. The nerve agents sarin, soman, and VX yield the same aged OP derivative, so that these agents may be difficult to distinguish when bound to acetylcholinesterase or butyrylcholinesterase [32,33].

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Three of the OP agents studied in this work—sarin, DFP, and chlorpyrifos oxon—are known to age when bound to acetylcholinesterase and butyrylcholinesterase. However, these OP toxicants did not age when bound to albumin. Aging is a catalytic process that requires the participation of nearby histidine and glutamic acid residues [34]. Residues that promote aging are not present in the active site pocket of albumin. The absence of aging allows the bound OP to be spontaneously released from Tyr411. This makes albumin an OP hydrolase, albeit a very slow one. Albumin hydrolyzes chlorpyrifos oxon, *O*-hexyl *O*-2,5-dichlorophenylphosphoramidate, and paraoxon [10–13]. We recently measured the hydrolysis of soman by human albumin and found a deacylation rate of 0.0052 per hour (unpublished).

The observation that OP–albumin adducts do not age is supported by the findings of others [8,9]. Using liquid chromatography–mass spectrometry (LC–MS), Black and coworkers found *O*-(pinacolyl methylphosphonyl)tyrosine in human plasma as well as in albumin samples that had been treated with soman, and they found *O*-(isopropyl methylphosphonyl)tyrosine in samples treated with sarin [8]. If aging had occurred, the products would have been (methylphosphonyl)tyrosine for both soman and sarin. Adams and coworkers used gas chromatography–mass spectrometry (GC–MS) to measure sarin and soman recovered from human plasma and albumin samples [9]. The plasma and albumin were reacted with sarin or soman, excess agent was removed by solid phase extraction, and the samples were treated with potassium fluoride to release the bound OP. Intact sarin and soman were recovered, demonstrating that aging had not occurred. The absence of aging in OP–albumin adducts suggests that albumin could be a useful biomarker to distinguish between soman and sarin exposure. In the same manner, OP–albumin adducts could distinguish between pesticide and nerve agent exposure.

Advantages and disadvantages of MALDI-TOF–MS

The MALDI-TOF–mass spectrometer is an easy instrument to use. Samples need to be free of salt, and the concentration of peptide needs to be approximately 1 pmol/μl. As little as 0.5 μl of a 1-pmol/μl solution gives a good signal. Results are acquired in seconds.

The disadvantage of MALDI is that not all peptides ionize when the sample contains a mixture of peptides. For example, the FP-biotin-labeled bovine albumin YTR peptide gave an intense signal at 1012 amu (data not shown). In contrast, FP-biotin-labeled human albumin YTK peptide gave no signal. In both experiments, the sample was a mixture of tryptic peptides. Ion suppression is a common problem in MS, and one way of solving the problem is to separate the peptide of interest from other peptides by HPLC before examining it by MALDI–TOF MS. This strategy allowed us to detect sarin-labeled albumin in human plasma. Alternatively, the peptides can be separated by step elution from a C18 ZipTip, a procedure that was successful for nerve agent adducts of acetylcholinesterase [35].

OP–albumin as a biomarker of OP exposure

Many proteins in human plasma are labeled by OP. MS assays have been developed for OP–butyrylcholinesterase adducts [36–38]. The current article has provided an assay for OP–albumin adducts. Albumin is far less reactive with OP than ~~with~~ butyrylcholinesterase, but the 10,000-fold higher concentration of albumin in plasma compared with butyrylcholinesterase (40,000–50,000 vs. 4–5 mg/ml) means that both albumin and butyrylcholinesterase will be labeled when a person is exposed to OP. New assays that use precursor and fragment ion *m*/*z* values in selected reaction monitoring experiments are expected to be capable of diagnosing low-dose exposure [38].

Antibody to OP–albumin

The information presented in this article could have application to the monitoring of individuals exposed to OP. It may be possible to detect exposure through the use of an antibody detection assay directed toward the OP–albumin adduct at Tyr411. There is precedent for the generation of antibodies to very small haptens bound to protein. For example, antibodies that distinguish among phosphotyrosine, phosphoserine, and phosphothreonine have been successfully produced [39,40]. Antibodies to soman, sarin, and VX bound to carrier proteins through a chemical linker have been produced [41–43]. The proposed OP–albumin epitope could be more useful for detection of OP exposure than ~~are~~ existing antibodies because the OP–albumin adduct has no chemical linker and no foreign protein environment.

An OP–albumin adduct at Tyr411 may generate an antibody response in exposed individuals, and the antibody could be detected to determine a history of exposure to OP. This would facilitate monitoring exposure to OP long after the exposure incident and long after the antigen has disappeared.

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